is to be attributed to a simple reversal of an equilibrium by addition of one of the products of the reaction, that is, the principle of Le Chatelier, rather than to some mysterious "stabilization" of the protein.

Registry No. IPTG, 367-93-1; ONPF, 1154-94-5.

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Native-like Folding Intermediates of Homologous Ribonucleases†

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ABSTRACT: The mechanism of the slow refolding reactions of four different pancreatic ribonucleases from ox, sheep, red deer, and roe deer has been investigated. Refolding kinetics of these proteins were very similar. In particular, a native-like intermediate, I_N, was shown to be populated on the slow refolding pathway of all ribonucleases. We conclude that, similar to the stability of the folded proteins, the pathway of slow refolding has been conserved despite the differences in amino acid sequence and the varying number of proline residues.

The three-dimensional structure of a folded polypeptide chain as well as the pathway of folding from the unfolded state to the native state is determined by the amino acid sequence of the protein (and by the native environment). The mechanism by which changes in primary sequence influence the pathway of folding can in principle be elucidated by a comparison of different mutant proteins from one species or of homologous proteins from different species, which have a similar three-dimensional structure but vary in the amino acid sequence (Crisanti & Matthews, 1981; Nall, 1983; Elwell & Schellman, 1979; Krebs et al., 1983; Hollecker & Creighton, 1983; Hawkes et al., 1984). The refolding characteristics of RNases[1] from ox, sheep, red deer, and roe deer have been examined recently and found to be very similar, despite the differences in primary sequences, which range from 4 to 17 substitutions (out of a total of 124 amino acids). In addition, the relative amplitudes of the fast and the slow refolding reactions were independent of the number of proline residues in the sequence (Krebs et al., 1983). These results suggested that the pathway of refolding, i.e., the sequence of intermediates and activated states on the pathway of folding, has been conserved during the evolution of these proteins. The mechanism of the major slow refolding reaction of bovine RNase A, which involves both folding and proline isomerization steps, has been characterized in considerable detail. Two intermediates of refolding have been detected on the slow refolding pathway: an early hydrogen-bonded intermediate, I_1 (Nall et al., 1978; Schmid & Baldwin, 1979; Kim & Baldwin, 1980), and a late native-like intermediate, I_N, which is compactly folded but still contains at least one incorrect proline isomer (Cook et al., 1979; Schmid & Blaschek, 1981). I_N accumulates transiently when folding is carried out under strongly native folding conditions (Schmid, 1983).

Here we ask whether the pathway of slow refolding of RNase A has been conserved. The RNases from sheep, red

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[1] Abbreviations: RNase, pancreatic ribonuclease (EC 3.1.27.5) with disulfide bonds intact; U_S^I, U_S^{II}, and U_F, slow- and fast-folding species of unfolded RNase; N, native RNase; I_1 and I_N, folding intermediates; 2′,3′-CMP, cytidine 2′,3′-phosphate; Gdn·HCl, guanidine hydrochloride; τ, time constant of a chemical reaction (reciprocal of the apparent rate constant, k^{-1}).

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deer, and roe deer were isolated, and the kinetics of slow refolding of these proteins were characterized and compared with the folding of the bovine enzyme. In particular, slow folding was investigated under strongly native conditions, where the presence of native-like folding intermediates is detected most readily.

Materials and Methods

Materials

Bovine pancreatic RNase A (type XII-A, lots 49C-8047 and 71F-8145) and 2′,3′-CMP were from Sigma (St. Louis, MO), Gdn·HCl (ultrapure) was from Schwarz/Mann (Orangeburg, NY), sodium cacodylate was from Fluka (Buchs, Switzerland), and Servacel CM52 carboxymethylcellulose was from Serva (Heidelberg, FRG). All other chemicals (analytical grade) were from Merck (Darmstadt, FRG).

Methods

Isolation of RNases from Sheep, Red Deer, and Roe Deer. The RNases were isolated following the methods of Åquist & Anfinsen (1959) and Zwiers et al. (1973) with several modifications. After acid extraction of the pancreatic tissue and fractionation by ammonium sulfate two consecutive chromatographic steps on CM-cellulose at pH 4–7 and at pH 8, respectively, were used to separate RNase from other pancreatic proteins. The isolated RNases showed single bands in sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Assays for carbohydrate were negative. For details of the isolation procedure see Krebs (1983). Concentrations of the various RNases were determined spectrophotometrically (Sela & Anfinsen, 1957; Krebs et al., 1983).

Slow Folding Kinetics. A Cary 118C spectrophotometer, a Hitachi Perkin-Elmer MPF-44 fluorescence spectrophotometer, and a Jasco J-500A spectropolarimeter with a DP-500N data processor were employed for kinetic experiments. Refolding was initiated by a 20-fold dilution of unfolded proteins (in 0.1 M glycine and 4.0 M Gdn·HCl, pH 2) to the desired final folding conditions. Final concentrations were in the range of 30 μM. Unfolding assays for I_N and N molecules were performed as described by Schmid (1983).

Results

Spectral Properties. Circular dichroic (CD) spectra in the amide region of all four RNases are virtually identical, suggesting that their backbone conformations are similar. This agrees with the conclusion that the amino acid sequences of the sheep and the deer enzymes are reasonably compatible with the three-dimensional structure of the bovine enzyme (Lenstra et al., 1977). Significant differences arise in the aromatic region of the CD spectra. Whereas the ovine enzyme shows the same spectrum as bovine RNase, the ellipticity of the deer RNases is decreased by about 15% in the 250–300-nm region, and these enzymes lack the positive ellipticity around 240 nm. These differences probably originate from the lack of Tyr-76 in the deer enzyme.

Tyrosine fluorescence of native RNase A is decreased because of hydrogen bonding of phenolic hydroxyl groups and the proximity of disulfide bonds in the folded molecule (Cowgill, 1967). The ovine enzyme (cf. Figure 1A) shows fluorescence properties identical with those of bovine RNase A; i.e., upon refolding, tyrosine fluorescence is quenched to about 40% of the emission of the unfolded protein. In contrast, the two deer enzymes show virtually no fluorescence in the native state; fluorescence is decreased to less than 10% compared to that of the unfolded protein in 5 M Gdn·HCl (Figure 1B). Probably most of the fluorescence intensity of native bovine and ovine RNase originates from Tyr-76. The substitution of this solvent-accessible tyrosine in the deer enzymes leads to the pronounced decrease of fluorescence described above. However, small changes in the native environment of the conserved tyrosine residues might lead to altered fluorescence properties as well.

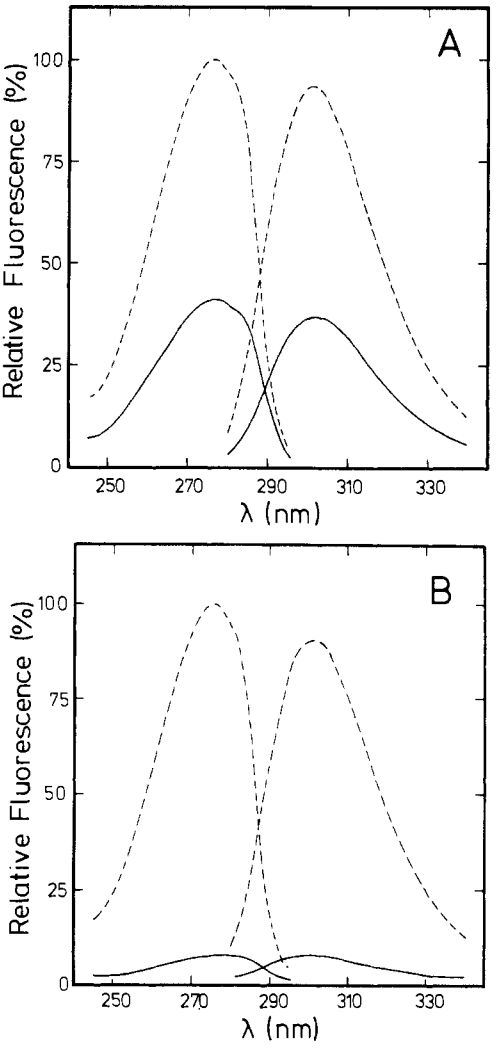

FIGURE 1: Fluorescence spectra of (A) ovine RNase A and (B) red deer RNase. Excitation spectra were recorded at a fixed wavelength of emission at 305 nm; for emission spectra the excitation wavelength was at the isosbestic point of native and unfolded protein at 268 nm. Spectra of the native proteins (—) were measured in 0.04 M ammonium acetate, pH 4.3 at 20 °C, and spectra of the unfolded samples (- - -) were measured under the same conditions with 5 M Gdn·HCl added.

Gdn·HCl-Induced Unfolding. (A) Equilibrium Unfolding Transitions. The various RNases display virtually identical stability toward unfolding induced by Gdn·HCl. Figure 2 gives a comparison of transition curves for the ovine and the red deer enzymes. Unfolding was reversible as judged by tyrosine absorbance as well as by the complete regain of enzymatic activity. The midpoints and the sizes of the Gdn·HCl-induced unfolding transitions measured at 10 °C and pH 6 are summarized in Table I. The absolute value of the absorbance at 287 nm (cf. Figure 2) as well as the change in absorbance upon unfolding is decreased for the deer enzymes presumably due to the lack of Tyr-76. In native RNase A Tyr-76 is the most accessible tyrosine residue; nevertheless, it appears to contribute significantly to the decrease in absorbance upon unfolding. Transition curves measured by the increase in

Table I: Comparison of Folding Properties of RNases under Investigation[a]

	species			
	ox	sheep	red deer	roe deer
unfolding transition at pH 6.0				
$[Gdn{\cdot}HCl]_{1/2}$[b] (M)	3.35	3.35	3.25	3.45
$\Delta\epsilon_{287}$[c] (M^{-1} cm^{-1})	2700	2870	2320	2440
unfolding kinetics at 4.7 M Gdn·HCl, pH 6.0				
τ (s)	180	120	120	150
$\Delta\epsilon_{287}$ (M^{-1} cm^{-1})	2710	2740	2320	2390
fluorescence-detected isomerization of the unfolded proteins[d]				
τ (s)	180	160	160	120
ΔF[e] (%)	15	16	18	18
ΔF_{cor}[f] (%)	15	17	16	15
slow refolding at 2.0 M Gdn·HCl, pH 6.0				
monitored by absorbance, τ (s)	870	690	790	680
monitored by fluorescence, τ (s)	860	nd	780	720
slow refolding at 0.2 M Gdn·HCl, pH 6.0				
monitored by absorbance				
τ_1 (s)	170	170	130	150
τ_2 (s)	29	27	13	25
f_2[g]	0.83	0.82	0.68	0.67
monitored by fluorescence				
τ_1 (s)	200	180	340	160
τ_2 (s)	45	45	100	59
f_2[g]	0.82	0.84	0.78	0.60
slow refolding at 0.2 M Gdn·HCl plus 0.4 M $(NH_4)_2SO_4$, pH 6.0				
monitored by absorbance				
τ_1 (s)	75	87	108	152
τ_2 (s)	7	6	8	16
f_2[g]	0.73	0.78	0.69	0.76
monitored by fluorescence				
τ_1 (s)	130	76	274	142
τ_2 (s)	43	37	90	67
f_2[g]	0.79	0.69	0.70	0.62

[a] All measurements were carried out at 10 °C; buffers used were 0.1 M sodium cacodylate at pH 6.0 and 0.1 M glycine at pH 2.0. The final concentrations of RNase were in the range of 30 μM for all experiments. Unfolding experiments were carried out by a 10-fold dilution of native RNase (in H_2O) to the final unfolding conditions. Refolding experiments were performed by a 20-fold dilution of unfolded RNase (in 0.1 M glycine and 4.0 M Gdn·HCl, pH 2.0) to the final refolding conditions. Absorbance was measured at 287 nm; fluorescence emission was measured at 305 nm (10-nm slit) with excitation at 268 nm (4-nm slit). [b] Midpoint of the equilibrium unfolding transition induced by Gdn·HCl. [c] Total change of the molar absorbance at $[Gdn{\cdot}HCl]_{1/2}$ upon unfolding. [d] Measured after rapid unfolding ($N \rightarrow U_F$) at pH 2 in the presence of 6.3 M Gdn·HCl. Isomerization was monitored by the increase in tyrosine fluorescence. [e] Relative amplitude of the isomerization; the final fluorescence intensity is set at 100%. [f] Relative amplitude; the final fluorescence of the bovine enzyme is used as 100% reference for all four proteins. [g] Relative amplitude of the faster (τ_2) phase.

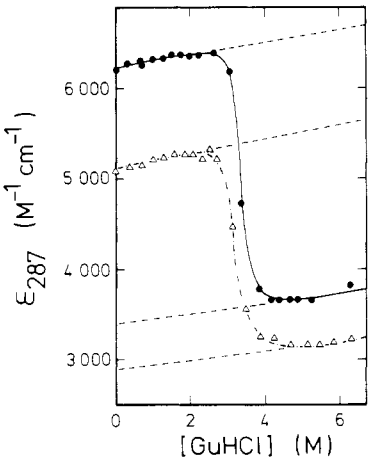

FIGURE 2: Comparison of the transition curves for ovine (●) and red deer (△) RNases. Unfolding induced by Gdn·HCl was monitored by the decrease in molar absorbance at 287 nm in 0.1 M sodium cacodylate, pH 6.0 at 10 °C.

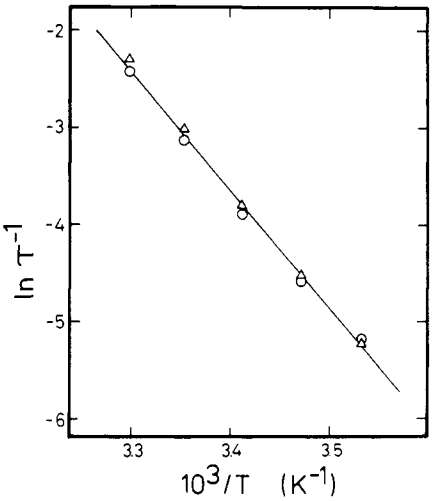

FIGURE 3: Temperature dependence of the rates of unfolding of bovine (△) and of roe deer (○) RNase A, measured in 4.7 M Gdn·HCl and 0.1 M sodium cacodylate, pH 6.0. The slope of the straight line yields an activation energy of 25 kcal/mol.

fluorescence are superimposable with the absorbance-detected unfolding curves.

(*B*) *Kinetics of Unfolding.* Unfolding kinetics were measured by absorbance (287 nm) at 10 °C, pH 6, and 4.7 M Gdn·HCl (Table I). Under these conditions for each enzyme, unfolding occurs in a single slow reaction that accounts for the entire change in absorbance at 287 nm. The rates of unfolding are similar (τ = 150 ± 30 s; cf. Table I). The activation energies for unfolding at pH 6 and 4.7 M Gdn·HCl were determined for the bovine and the roe deer enzymes: both unfolding reactions are governed by an activation energy of 25 ± 1 kcal/mol (Figure 3).

The mechanism of unfolding of bovine RNase A depends on pH. At neutral pH unfolding ($N \rightarrow N_F$) is a slow sin-

gle-phase reaction, which is followed by the $U_F \rightleftharpoons U_S$ isomerization reaction of the unfolded chain (eq 1) (Garel & Baldwin, 1973; Schmid, 1985). At acidic pH unfolding is a sequential process, involving the intermediate I_U (eq 2) (Hagerman & Baldwin, 1976; Hagerman et al., 1979). Unfolding is rapid; the time constants of the steps leading to U_F are in the time range of milliseconds (Hagerman & Baldwin, 1976). A similar change in the mechanism of unfolding upon lowering the pH can be observed for the ovine and the deer RNases. In all cases, the rate of unfolding (as detected by the change in tyrosine absorbance) is strongly increased by lowering the pH and is complete within the time of manual mixing (5 s) at pH 2, 4 M Gdn·HCl, and 10 °C.

$$N \rightarrow U_F \rightleftharpoons U_S \quad (1)$$

$$N \rightleftharpoons I_U \rightleftharpoons U_F \rightleftharpoons U_S \quad (2)$$

Fluorescence Changes during the $U_F \rightleftharpoons U_S$ Equilibration. The $U_F \rightleftharpoons U_S$ reaction of unfolded RNase involves the isomerization of X-Pro peptide bonds (Brandts et al., 1975; Schmid & Baldwin, 1978; Lin & Brandts, 1983a). This equilibration is almost silent in tyrosine absorbance, but it is accompanied by an increase in fluorescence emission, which was interpreted to result from a local effect of the isomerization of the Tyr-92–Pro-93 peptide bond on the fluorescence properties of Tyr-92 (Rehage & Schmid, 1982; Lin & Brandts, 1983b). These fluorescence changes are detected most easily under conditions where the fast $N \rightarrow U_F$ and the slow $U_S \rightleftharpoons U_F$ isomerizations are kinetically decoupled (i.e., at acidic pH). Such a slow increase in fluorescence after rapid $N \rightarrow U_F$ unfolding was detected for all RNases under investigation (Table I). The corresponding amplitudes are the same in each case; they amount to about 16% of the emission of unfolded bovine RNase A. The involved time constants are also similar, and they correlate well with the time constants for the overall formation of U_S species as determined by the "double-jump" procedure (Krebs et al., 1983), although for every RNase investigated, the rate of the fluorescence changes after unfolding (Table I) is slightly higher than the overall rate of the $U_F \rightleftharpoons U_S$ reaction, which probably involves more than one isomerization of the unfolded chain (Lin & Brandts, 1983b).

Slow Refolding Kinetics of the Different Ribonucleases. The kinetics of the slow refolding reactions of the four RNases were monitored by the increase in absorbance and by the decrease in fluorescence upon folding. At 10 °C and pH 6, three different refolding conditions were employed to characterize and to compare the slow refolding reactions. These conditions varied from marginally stable (close to the onset of the unfolding transition at 2 M Gdn·HCl) to strongly native (in the presence of 0.4 M ammonium sulfate).

Refolding is very slow under conditions of marginal stability of the native protein. In the presence of 2.0 M Gdn·HCl slow refolding occurs in a single kinetic phase, and fluorescence- and absorbance-detected kinetics are governed by the same rate-limiting step. The observed time constants were in the range of 800 ± 100 s, for all RNases investigated (Table I). Neither intermediates of folding nor heterogeneity of the U_S species was detected under these conditions of marginal stability.

At a final concentration of 0.2 M Gdn·HCl (which is a "more native" condition) folding rates are increased (Table I). Both methods, absorbance and fluorescence, monitored two slow folding reactions that originate from the presence of at least two kinetically distinguishable populations of slow-refolding molecules, U_S^I and U_S^{II} (Schmid & Blaschek, 1981; Denton et al., 1982). Under these conditions the rates of related folding reactions of the different RNases vary within about a factor of 2, but still they share the common basic properties: there are two slow folding reactions, detected by both absorbance and fluorescence; the relative amplitude of the faster phase amounts to 70–80%; and the fluorescence-detected folding tends to be slightly slower than absorbance-detected kinetics.

Under strongly native conditions (e.g., in the presence of ammonium sulfate) a native-like intermediate was shown to accumulate on the major slow $U_S^{II} \rightarrow N$ refolding pathway of bovine RNase A (eq 3) (Cook et al., 1979; Schmid & Blaschek, 1981). I_N is similar to the native protein in absorbance and in its enzymatic properties; the fluorescence emission of I_N, however, is still largely unquenched. Absorbance-detected formation of I_N ($U_S^{II} \rightarrow I_N$) is strongly accelerated by the addition of ammonium sulfate, whereas the final $I_N \rightarrow N$ step (monitored by the decrease in fluorescence) is independent of ammonium sulfate concentration. In the presence of 0.4 M ammonium sulfate the slow refolding kinetics of all RNases (Table I) are biphasic with a major faster and a minor slower phase, reflecting the $U_S^{II} \rightarrow N$ and the $U_S^I \rightarrow N$ refolding pathways, respectively. For all RNases, absorbance changes rapidly in the $U_S^{II} \rightarrow I_N$ step; this is followed by the slower $I_N \rightarrow N$ step, which is monitored by fluorescence. There are variations in the observed time constants for the individual proteins: the rate of the absorbance-detected $U_S^{II} \rightarrow I_N$ refolding step of the roe deer enzyme is decreased by a factor of about 2 relative to the bovine RNase A; fluorescence-detected refolding (i.e., the $I_N \rightarrow N$ step) is slowest for RNase from red deer.

$$U_S^{II} \rightarrow I_N \rightarrow N \quad (3)$$

Unfolding Assays for I_N Molecules. The slow refolding reactions of the different RNases under investigation are similar under strongly native conditions. This suggests that a native-like intermediate, I_N, which had been located on the folding pathway of bovine RNase A, is populated in the course of the major slow refolding reactions of the other RNases as well (see above). The transient presence of I_N molecules at any time point during refolding can be detected by unfolding assays. These assays make use of the large difference in the rates of unfolding of I_N and N molecules. I_N shows native-like tyrosine absorbance; however, it unfolds much more rapidly than native RNase (Schmid, 1983). Table II compares the unfolding kinetics of native RNase with the unfolding of protein samples that were exposed to a refolding pulse of 20 s at 0.2 M Gdn·HCl, pH 6, and 10 °C in order to populate I_N. Similar to the bovine enzyme, unfolding of native RNase from red deer and roe deer occurs in a single slow reaction, which accounts for the entire change in absorbance upon unfolding (cf. Figure 2). Unfolding of the samples that had been exposed to a 20-s refolding pulse resulted in two well-separated unfolding reactions for all three RNases investigated. The slow phases were caused by the unfolding of fully native protein; the fast phases originated from the rapid unfolding of the native-like intermediates I_N, which accumulated transiently during the 20-s folding pulse. These results (Table II) demonstrate that native-like intermediates are populated on the slow refolding pathways of the deer proteins and that the time constants for unfolding of I_N, τ_2, are similar for the three RNases. The I_N state is most strongly populated on the folding pathway of the red deer RNase. This was expected since absorbance-detected formation of I_N ($U_S \rightarrow I_N$ step) is fastest, and the decay of I_N detected by fluorescence changes ($I_N \rightarrow N$ step, cf. eq 3) is slowest for the red deer enzyme compared to the other RNases (cf. Table I).

Table II: Unfolding Properties of Native Proteins and of a Mixture of Native Protein and Native-like Intermediates[a]

	unfolding of native enzyme		unfolding after folding pulse to populate I_N[b]					
enzyme source	τ (s)	$\Delta\epsilon_t$[c] (M^{-1} cm^{-1})	τ_1 (s)	$\Delta\epsilon_1$[d] (M^{-1} cm^{-1})	N[e] (%)	τ_2 (s)	$\Delta\epsilon_2$[d] (M^{-1} cm^{-1})	I_N[e] (%)
ox	180	2710	180	1010	37	16	870	32
red deer	120	2320	120	700	30	11	1030	44
roe deer	150	2390	160	680	28	16	860	36

[a] Unfolding assays were carried out in 0.1 M sodium cacodylate and 4.7 M Gdn·HCl, pH 6.0, at 10 °C. [b] A mixture of I_N, N, and U_S molecules was populated by a 20-s refolding pulse at 0.2 M Gdn·HCl, pH 6.0, at 10 °C. (The RNases were initially unfolded in 4.0 M Gdn·HCl, pH 2.0.) [c] Molar decrease in absorbance at 287 nm upon unfolding of native RNase. [d] $\Delta\epsilon_1$ and $\Delta\epsilon_2$: absorbance changes accompanying the slow unfolding of N and the rapid unfolding of I_N, respectively. [e] Fractional change in absorbance upon unfolding of N and I_N, expressed as a percentage of the total change upon unfolding: % N = $100(\Delta\epsilon_1/\Delta\epsilon_t)$; % I_N = $100(\Delta\epsilon_2/\Delta\epsilon_t)$.

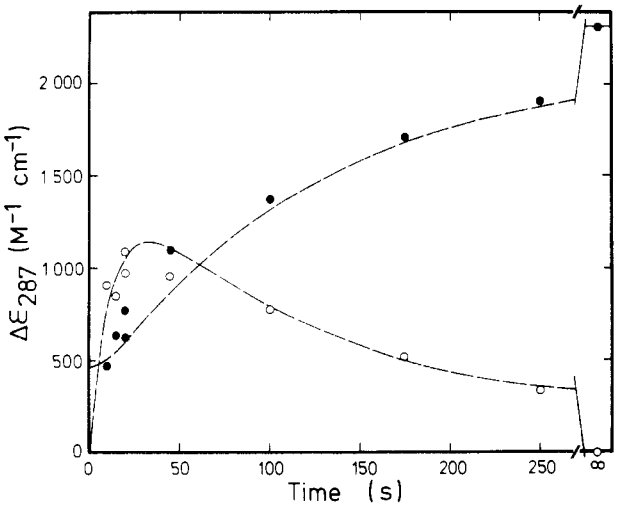

FIGURE 4: Refolding of RNase from red deer. Concentration vs. time curves for I_N (O) and N (●) during refolding at 0.2 M Gdn·HCl and 0.1 M sodium cacodylate, pH 6 at 10 °C. The amplitudes of the unfolding assays (carried out at 0.1 M sodium cacodylate and 4.7 M Gdn·HCl, pH 6 at 10 °C) for I_N and N (expressed as molar changes) are shown as a function of the time of sample withdrawal from the refolding solution. The dashed lines are calculated for sequential refolding by using the time constants and fractional amplitudes of the two slow phases given in Table I. For the calculation it was assumed that the $U_F \rightarrow N$ reaction yields 20% of N molecules within the dead time of the experiment and that I_N is an intermediate on both slow refolding pathways.

Transient Formation of I_N during Slow Refolding. Unfolding assays for I_N and N can be used to detect these species at any time during folding, independently of each other, in order to probe the sequential nature of slow refolding according to eq 3 (Schmid, 1983). Refolding of RNase from red deer at 10 °C and 0.2 M Gdn·HCl, pH 6, was investigated by unfolding assays, and the kinetic curves for I_N and N are shown in Figure 4. As expected for a sequential reaction, the intermediate I_N accumulates transiently during refolding with a maximal population of I_N about 40 s after initiation of folding. A total of 20% of native RNase A (equivalent to $\Delta\epsilon_{287}$ = 0.20 × 2320 = 460 M^{-1} cm^{-1}) is formed rapidly during the fast $U_F \rightarrow N$ folding reaction. The subsequent slow increase in N molecules is paralleled by the concomitant decrease in the population of I_N. The lines drawn in Figure 4 are calculated for a sequential model as described by Schmid (1983) by using the time constants of Table I. The comparison of the results in Figure 4 with the transient formation of I_N molecules on the folding pathway of bovine RNase A (Schmid, 1983) confirms that the mechanism of slow refolding is essentially identical for the RNases from red deer and from bovine pancreas.

Reactivation Kinetics of Red Deer RNase. Refolding of the enzyme from red deer was probed by RNase activity assays under conditions where the $U_S \rightarrow N$ reaction is slow and occurs in a single first-order kinetic phase (1.2 M Gdn·HCl, pH 6.0, 10 °C). The activity assays were carried out at 10 °C in the presence of 2.5 M Gdn·HCl, in order to inhibit markedly further refolding during the assays. The results (Figure 5) show that reactivation (τ = 350 s) parallels refolding by the increase in tyrosine absorbance under identical conditions (τ = 380 s; data not shown). A total of 20% of the active molecules are formed rapidly in the dead time of the experiment (10 s) in the course of the fast $U_F \rightarrow N$ refolding reaction, indicating that at 10 °C unfolded RNase from red deer (like bovine RNase A) consists of a 20:80 mixture of fast- and slow-folding molecules. This agrees with earlier results of stopped-flow refolding experiments which showed that at 35 °C all investigated RNases displayed a common U_F:U_S ratio of 20:80 (Krebs et al., 1983).

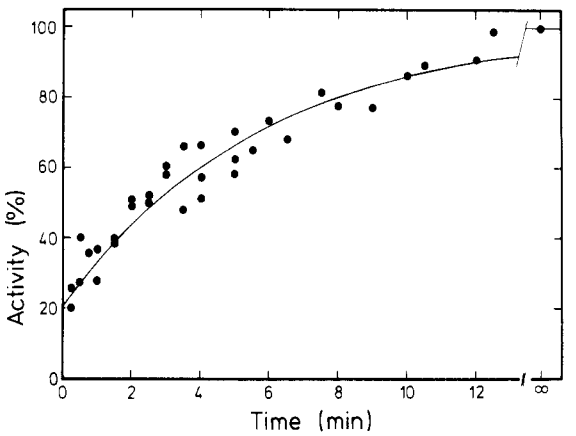

FIGURE 5: Time course of reactivation of red deer RNase at 1.2 M Gdn·HCl, pH 6 at 10 °C. Reactivation was initiated by a 16-fold dilution of unfolded protein (in 4 M Gdn·HCl and 0.05 M glycine, pH 1.8) to final 1.2 M Gdn·HCl, 0.1 M sodium cacodylate, pH 6, and 64 μM RNase. Aliquots were withdrawn and assayed for RNase activity in the presence of 0.34 mg/mL 2′,3′-CMP, 2.5 M Gdn·HCl, pH 7.0 at 10 °C, and 6.4 μM RNase. Under these conditions further refolding is strongly decelerated. The continuous line represents a single exponential curve with τ = 350 s and an amplitude of 80%.

Discussion

The Stability and the Mechanism of Slow Refolding of Different RNases Are Conserved. The RNases isolated from pancreatic tissue of ox, sheep, red deer, and roe deer show almost identical stability toward unfolding by Gdn·HCl. The midpoints of the transitions are at 3.35 ± 0.10 M Gdn·HCl; the cooperativities of the transitions are within experimental accuracy identical; $\Delta b_{23} = \partial(\ln K_{app})/\partial[\text{Gdn·HCl}]_{T,P}$ = 6.2 ± 0.4 [cf. Schellman (1978)]. This agrees with thermal unfolding transitions of these enzymes, which also display similar midpoints of unfolding (Krebs et al., 1983). Hence stability against thermal as well as denaturant-induced unfolding is not

affected by the amino acid substitutions.

Earlier work from this laboratory (Krebs et al., 1983) demonstrated that, in addition to similar stability, the investigated RNases follow the same overall mechanism of refolding; i.e., the ratio of fast- and slow-refolding species U_F:U_S is constant at 20:80, and the rates of the respective fast and slow refolding reactions are similar at 35 °C, pH 6, and 0.7 M Gdn·HCl. The detailed investigation of the slow $U_S \rightarrow N$ folding reaction presented here indicates that, in addition to the constant amount of U_F, the slow-folding U_S species of the investigated RNases are composed of a minor U_S^I and a major U_S^{II} species throughout. If proline isomerization reactions are responsible for the occurrence of multiple unfolded forms of a protein (Brandts et al., 1975; Lin & Brandts, 1983b), then this implies that the additional proline residues 15 and 17 of the deer enzymes influence neither the distribution between these different unfolded species nor the rate at which they refold. This agrees with earlier conclusions that proline residues exist which may be nonessential for folding (Babul et al., 1978; Levitt, 1981; Jullien & Baldwin, 1981). The mechanism of slow refolding is strongly conserved for the investigated RNases under marginally stable conditions as well as under strongly native conditions. In particular, native-like intermediates I_N accumulate on the folding pathways of all four RNases. These intermediates are closely related or identical in their structural and kinetic properties despite the differences in amino acid sequence and the varying number of proline residues.

No definitive conclusions regarding the presence of early intermediates I_1 (Nall et al., 1978; Schmid & Baldwin, 1979; Kim & Baldwin, 1980) can be drawn. However, the similarity of the kinetics of I_N formation, in particular their strong dependence on the conditions of folding, suggests that formation of early I_1-like intermediates controls the slow refolding of the RNases from sheep, red deer, and roe deer as well.

The amino acid sequences of about 40 homologous mammalian RNases have been determined to date (Blackburn & Moore, 1982; Beintema & Lenstra, 1982; Beintema & Neuteboom, 1983; Beintema et al., 1984). In general, the sequence is most strongly conserved within the elements of secondary structure, i.e., the α-helices and the β-strands. There is one exception to this pattern, namely, the β-strand 97–104, where frequent amino acid substitutions occur. Those parts of the sequence where maximal variability occurs are the loop regions 15–23 and 31–39. More than half of the differences in sequence between the bovine RNase and the deer RNases occur in these three limited regions of the protein molecule. As there are no significant differences in the folding of these RNases, we tentatively conclude that these parts of the polypeptide chain are not critically important for the pathway of RNase folding. Two other differences in sequence involve substitutions of aromatic residues: Ser-59 of bovine and ovine RNase is replaced by phenylalanine in the deer proteins, and Tyr-76 is substituted by Asn-76. Both positions are accessible to solvent in bovine RNase (Richards & Wyckoff, 1973), and therefore folding is not likely to be influenced by these changes. There is only one substitution of a completely buried residue; i.e., Met-35 of red deer RNase is replaced by Leu-35 in the other RNases. This conservative substitution probably does not change the stability of folded structures.

Folding Intermediates Are Probably Stabilized by Similar Interactions As in the Native Protein. Our results provide evidence that amino acid substitutions in homologous RNases which do not change the stability of the native, folded protein also do not affect markedly the stability of folding intermediates and the energy of activated states between them. There is no evidence in our data that folding intermediates exist that are characterized by stabilizing interactions no longer present in the native state (Lim, 1980; Goldenberg et al., 1983). The investigation of the refolding kinetics of homologous cytochromes *c* that differ in stability (Brems et al., 1982; Brems & Stellwagen, 1983; Nall & Landers, 1981; Zuniga & Nall, 1983) indicates that the rates of folding depend on the stability of the native protein. At equal distances from the midpoints of Gdn·HCl-induced unfolding of the native proteins the rates of refolding of the various cytochromes *c* are almost identical (Brems et al., 1982). This correlation again indicates that the stability of folding intermediates and of activated states that control the rates of folding is closely related to the stability of the native state.

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Probing Different Conformational States of Bovine α-Lactalbumin: Fluorescence Studies with 4,4′-Bis[1-(phenylamino)-8-naphthalenesulfonate]†

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ABSTRACT: The binding of the fluorescent probe 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate] (bis-ANS) to bovine α-lactalbumin (α-LA) was investigated. A strong dependence of the K_d value with the bound calcium stoichiometry was found, with K_d values ranging from 6.2 ± 0.4 to 64.6 ± 5.9 μM for apo-α-LA and 1:1 Ca(II)–α-LA, respectively. A 350-fold enhancement of the bis-ANS emission was observed in the protein–bis-ANS complex, along with an ~33-nm blue shift. Both appeared to be related to the hydrophobicity of the binding site and were *independent* of the Ca(II) ion content. From the difference in bis-ANS affinity between apo-α-LA and Ca(II)–α-LA, we demonstrated that Zn(II) and Al(III) were able to "lock" the protein into a new "apo-like" conformation, which was *similar* to, but not *identical* with, the apo conformation. The protein could be interconverted between all three conformations in a Mn(II) titration. The first Mn(II) shifted the apoprotein to the Ca(II) conformation; at higher Mn(II) levels, binding to the second site shifted the protein toward the apo-like conformation. The same behavior was observed with calcium in *large* excess. The evidence supported a model for the mutually nonexclusive binding of metals both to site I ("calcium site") and to site II ("zinc site") simultaneously. The results suggest that α-lactalbumin possesses a hydrophobic surface that becomes somewhat less accessible upon 1:1 calcium binding in the *absence* of metals that also bind to the zinc site.

The protein α-lactalbumin (α-LA)[1] is the modifier of the "lactose synthase" complex (UDPgalactose:D-glucose 4-β-D-galactosyltransferase, EC 2.4.1.22). Upon binding to the enzyme galactosyltransferase (EC 2.4.1.38), the catalytically active complex specifies the synthesis of lactose by galactosyl transfer to acceptor glucose, instead of terminal GlcNAc residues. Although putative three-dimensional structures of α-LA have been inferred from its high primary structural homologies to that of hen egg white lysozyme (Browne et al., 1969; Warme et al., 1974), no crystal structure has been reported to date.

A primary objective of our research has been to understand how α-LA interacts with galactosyltransferase, i.e., what region of the α-LA structure is involved in this interaction, the metal ion requirements for complex formation under physiological conditions, and whether any conformational transitions occur during the binding of α-LA to galactosyltransferase. It is known from previous studies that apo-α-LA undergoes a conformational change upon calcium binding or upon zinc binding to the Ca(II) form at a different, but distinct site (Murakami et al., 1982; Murakami & Berliner, 1983). Lindahl & Vogel (1984), using phenyl-Sepharose affinity chromatography, recently suggested that a hydrophobic surface is exposed in apo-α-LA that disappears upon calcium binding,

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[1] Abbreviations: bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]; α-LA, α-lactalbumin; GlcNAc, *N*-acetyl-D-glucosamine; ANS, 1-(phenylamino)-8-naphthalenesulfonate; EDTA, ethylenediaminetetraacetic acid.

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